

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representation of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

## **IMAGES ARE BEST AVAILABLE COPY**

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

THIS PAGE BLANK (USPTO)



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 3 :  G01N 33/54		A1	(11) International Publication Number: <b>WO 84/ 02579</b>  (43) International Publication Date: 5 July 1984 (05.07.84)		
(21) International Application Number: PCT/US83/02047		(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), JP, LU (European patent), NL (European patent), SE (European patent).			
(22) International Filing Date: 21 December 1983 (21.12.83)					
(31) Priority Application Number: 452,798					
(32) Priority Date: 23 December 1982 (23.12.82)					
(33) Priority Country: US		Published <i>With international search report.</i>			
(71) Applicant: COOPER-LIPOTECH, INC. [US/US]; 1030 Curtis Street, Menlo Park, CA 94025 (US).					
(72) Inventors: MARTIN, Francis, Joseph ; 415 West Portal Avenue, San Francisco, CA 94127 (US). HUERTAS, Pedro, Enrique ; 1521 Alameda, Alameda, CA 94501 (US).					
(74) Agents: WATT, Phillip, H. et al.; Fitch, Even, Tabin & Flammery, Room 900, 135 South LaSalle Street, Chicago, IL 60603 (US).					

(54) Title: LIPID-VESICLE-SURFACE ASSAY REAGENT AND METHOD

## (57) Abstract

An improved enzyme immunoassay reagent composed of lipid vesicles coated with a mobile surface array of ligand and enzyme molecules. The reagent is adapted for use in an enzyme immunoassay in which the reagent partitions between a separable support and a liquid phase, in proportion to the amount of analyte present. The amount of reagent in the liquid or support phase is determined by measuring the enzyme activity associated with the reagent in that phase. Also described is an immunoassay employing such a reagent.

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	LI	Liechtenstein
AU	Australia	LK	Sri Lanka
BE	Belgium	LU	Luxembourg
BR	Brazil	MC	Monaco
CF	Central African Republic	MG	Madagascar
CG	Congo	MR	Mauritania
CH	Switzerland	MW	Malawi
CM	Cameroon	NL	Netherlands
DE	Germany, Federal Republic of	NO	Norway
DK	Denmark	RO	Romania
FI	Finland	SE	Sweden
FR	France	SN	Senegal
GA	Gabon	SU	Soviet Union
GB	United Kingdom	TD	Chad
HU	Hungary	TG	Togo
JP	Japan	US	United States of America
KP	Democratic People's Republic of Korea		

-1-

LIPID-VESICLE-SURFACE ASSAY REAGENT AND METHOD

Background and Summary

The following publications are referred to by

5 corresponding number in this application:

1. Szoka, F. Jr., and Papahajopoulos, D., Ann. Rev. Biophys. Bioeng., 9:467-508 (1980).
2. Szoka, F., Jr. and Papahajopoulos, D., Proc. Nat. Sci. USA, 75:4194-4198 (1978).
- 10 3. Heath, T. D., Macher, B. A. and Papahadjopoulos, D., Biochimica et Biophysica Acta, 640:66-81 (1981).
4. Martin, F. J., Hubbell, W. L., and Papahadjopoulos, D., Biochemistry, 20:4229-4238 (1981).
- 15 5. Martin, F. J. and Papahadjopoulos, D., J. Biol. Chem., 257:286-288 (1982).
6. Curman, B., Klareskog, L., and Peterson, P. A., J. Biol. Chem., 255:7820-7826 (1980).
7. Smith, B. A. and McConnell, H. M., Proc. Nat. Acad. Sci. USA, 75, 2759-2763 (1978).
- 20 8. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193:265-275 (1951).

The present invention relates to a lipid-vesicle-surface assay reagent, and to enzyme immunoassay methods using such a reagent.

25 A variety of methods for determining the presence or concentration of biochemical analytes is known. The analyte to be assayed typically is one which plays an important role in biochemical processes. Low molecular weight substances, such as peptide and steroid hormones, vitamins and the like, and high molecular weight substances such as carbohydrates and proteins are commonly assayed analytes.

30 Several important analyte assay techniques are based on a reaction between the analyte and an anti-analyte capable of binding the analyte with high affinity and specificity. Typical analyte anti-analyte



-2-

binding pairs include antigen-antibody, immunoglobulin-protein A, carbohydrate-lectin, biotin-avidin, hormone-hormone receptor protein and complementary oligo- and polynucleotide strands. The 5 terms "ligand" and "antiligand" will be used herein to designate the opposite binding members in such a binding pair.

Among the various types of assays which employ specific binding reactions, enzyme immunoassays provide 10 a number of advantages in sensitivity, low cost and simplicity. In one type of enzyme immunoassay, an enzyme-ligand reagent is reacted in the presence of a ligand or ligand-like analyte with a solid support having anti-ligand binding sites carried on its surface, 15 wherein the analyte and ligand-enzyme reagent compete for binding to the solid support. In another type of enzyme immunoassay, a ligand analyte is capable of binding both to anti-analyte binding sites on a solid support, and to an anti-ligand in a anti-ligand-enzyme 20 reagent, to couple the reagent to the support in a sandwich fashion. In both assay types, the amount of analyte present is determined by separating the liquid and solid (support) phases, and measuring the enzyme 25 activity associated with one or both phases. Where the reagent and analyte compete for binding sites on the solid support, the enzyme activity associated with the support is inversely proportional to the concentration of analyte present. On the other hand, a direct 30 relationship between the amount of analyte and the enzyme activity associated with the solid support is observed where the analyte functions to join the reagent to the support by sandwiching.

Commonly, the reagent used in an enzyme immunoassay test includes an enzyme molecule covalently 35 coupled to a ligand or anti-ligand molecule to form a bimolecular pair. This type of reagent has limited



-3-

sensitivity, inasmuch as each reagent binding event is "reported" by one enzyme molecule only. This limitation has prevented general application of the enzyme immunoassay technique to cell typing based on the 5 detection of selected cell surface antigens, except in cases where antigen surface concentrations are quite large. Another limitation is that the bimolecular reagent must be formed from a relatively pure ligand preparation. Otherwise, a significant portion of the 10 reagent (the portion composed of enzyme coupled to non-ligand molecules) will not bind, or will bind nonspecifically, to the solid support. As a result, a high background attributable to unbound reagent (in the liquid phase) and nonspecifically bound reagent (in the 15 solid phase) will be observed.

An enzyme immunoassay reagent composed of lipid vesicles coated with ligand molecules and encapsulating enzymes within the interior vesicle spaces has been proposed in the prior art. A reagent of this type may 20 be relatively expensive to manufacture due to the recognized problems of encapsulating enzymes within liposomes efficiently. Further, many enzymes appear to undergo loss of activity during encapsulation (reference 1). The encapsulating vesicles must be lysed before 25 enzyme activity associated with the vesicles can be measured. Complement has been used for lysing lipid vesicles, but this method often lacks reproducibility due to complement inactivation on storage. Detergent lysis has been used, but this approach may be unsuitable 30 for applications --e.g., cell typing-- where the cell "support" to which the vesicles are bound is itself susceptible to detergent lysis.

It is therefore one general object of the present invention to provide, for use in an enzyme 35 immunoassay, a reagent which overcomes the above limitations associated with prior art enzyme immunoassay



-4-

reagents.

A more specific object of the present invention is to provide such a reagent composed of particles which have been prepared to have a highly mobile, or fluid 5 surface array of enzyme and ligand molecules.

Yet another object of the invention is to provide such a reagent composed of lipid vesicles which, in the .05 to 10 micron diameter range, each has an average of at least about 15 ligand molecules and up to 10 several thousand enzyme molecules attached to its surface.

Another important object of the invention is to provide, for use in an enzyme immunoassay method, a reagent capable of giving a high signal-to-noise ratio 15 in the method.

A related object of the invention is to provide an enzyme immunoassay kit containing such a reagent, and a separable support having surface binding site molecules which may be directly bound to the support or 20 may be carried on lipid vesicles which are themselves attached to the support surface.

Still another object of the invention is to provide such a reagent which can be used in a wide variety of different enzyme immunoassay methods.

25 The reagent of the invention is composed of lipid vesicle particles having a highly mobile, or fluid surface array of ligand and enzyme molecules. The particles are preferably lipid vesicles in the 0.05 to 10.0 micron diameter size range, and include an average 30 of at least about 15 ligand molecules and up to several thousand enzyme molecules bound to each vesicle surface. The ligand molecules may include one or more substantially pure ligand species, or may include impure mixtures thereof.

35 The method of the invention includes reacting the reagent with a separable support to produce



-5-

partitioning of the reagent between the support and liquid reaction phases, according to the concentration of analyte present.

5 The invention also contemplates an assay kit including a separable support and the reagent.

These and other objects and features of the invention will become more fully apparent from the following detailed description of the invention.

Detailed Description of the Invention

10 Preparation of Lipid Vesicles

The assay reagent of the present invention is composed of closed lipid vesicles, each having attached to its outer surface a laterally mobile array of enzyme and ligand molecules. Typically, the lipid vesicles 15 take the form of lipid bilayer structures encapsulating an aqueous interior region, such structures also being referred to as liposomes. The properties and methods of preparation of lipid vesicles have been detailed in the literature. The reader is referred particularly to 20 above-numbered references 1 and 2, and references cited therein, for a comprehensive discussion of the topic. What will be described herein are preferred methods of preparing liposomes used in forming the reagent of the invention, and liposome properties which contribute to 25 the advantages of the invention.

Lipid vesicles are prepared from lipid mixtures which typically include phospholipids and sterols. A list of phospholipids used typically in liposome preparations is given on page 471 of reference 1. One 30 consideration which determines the choice of lipids used is the degree of fluid mobility and lipid packing density which is desired in the vesicles formed. As reported in a number of literature reports, these characteristics can be varied according to the lengths 35 and degree of saturation of the aliphatic chains in the lipids, and the ratio of sterol to aliphatic chain



lipids used. The significance of surface fluid mobility in the the vesicle reagent of the invention will be seen below. Packing density characteristics are important to the success of reactions used to attach ligand and 5 enzyme molecules covalently to the vesicle surfaces. For example, it has been found that the inclusion of at least about 10% mole per cent of cholesterol is important for the success of certain protein-coupling reactions which will be described below. The fluidity 10 and packing characteristics will also affect the size and number of bilayers in the vesicles produced.

The vesicle lipid composition is also selected to produce a requisite number of specific lipid head groups through which the surface-bound reagent 15 components can be coupled to the vesicles. The head groups, or necessary modifications thereof, may be formed in the prepared liposomes, or in the individual lipids before incorporation into the liposomes. Examples of lipids used in preferred coupling reactions 20 will be discussed below.

The number of and type of polar lipid groups may also be selected to produce a desired charge distribution on the lipid vesicles at a selected pH and ionic strength. The charge distribution may affect the 25 relative reactivities of enzyme and ligand molecules in their coupling to lipid vesicles, as will be seen in Example III, and is an important feature in minimizing non-specific binding of the reagent vesicles to charged solid supports, as will be discussed.

A typical lipid composition used in preparing 30 lipid vesicles for the reagent of the invention preferably includes between about 10 and 50% cholesterol or other sterol, between about 2 and 50% of glycolipid or phospholipid to which the enzyme and ligand molecules 35 of the reagent can be individually coupled, with the remainder lipid composed of a neutral phospholipid, such



-7-

as phosphatidylcholine, or a phospholipid mixture. Charged lipids, such as phosphatidylserine, phosphatidic acid, glycolipids and charged cholesterol derivatives such as cholesterol hemisuccinate or cholesterol sulfate may be included to produce a desired surface charge in the lipid vesicles.

The lipid vesicles may be formed by one of a variety of methods discussed particularly in reference 1. Multilamellar vesicles --that is, vesicles composed of a series of closely packed bilayer lamellae-- can be prepared by drying a mixture of lipids in a thin film and hydrating the lipids with an aqueous buffer. The size and number of lamellae in the formed lipid vesicles can be controlled, within limits, by varying the 15 hydration time and amount of agitation used in hydrating the lipids. Where desirable, vigorous agitation, brief sonication or extrusion through polycarbonate membranes can be employed to obtain smaller and more uniformly sized multilamellar vesicles.

Small unilamellar vesicles having diameters of about 0.05 micron or less can be formed by sonicating a suspension of large multilamellar vesicles, either by probe or bath sonication. Another technique for producing small unilamellar vesicles involves the 25 removal of detergent from a detergent-phospholipid mixture by dialysis. Typical detergents include cholate and deoxycholate. An alternative method for the preparation of small unilamellar vesicles that avoids both sonication and detergents employs an ethanol injection step in which lipids dissolved in ethanol are rapidly injected into a buffer solution. A similar 30 technique in which phospholipids dissolved in ether-containing solvents has been used to produce large unilamellar vesicles with a generally heterogenous size 35 distribution.

In one preferred method of preparing large



-8-

unilamellar vesicles, referred to as reverse phase evaporation, a desired composition of lipids is dissolved in a suitable organic solvent such as diethyl ether, isopropyl ether, or a solvent mixture such as 5 isopropyl ether and chloroform (1:1). An aqueous solution is added directly to between about 3 and 6 volumes of the lipid-solvent mixture and the preparation is sonicated for a brief period to form a homogeneous emulsion. The organic solvent, or solvent mixture is 10 removed under reduced pressure, resulting in the formation of a viscous, gel-like intermediate phase which spontaneously forms a liposome dispersion when residual solvent is removed by evaporation under reduced pressure. The size of the resulting vesicles may be 15 varied according to the amount of cholesterol included in the lipid mixture. The reader is referred to references 1 or 2 for further details concerning the reverse phase evaporation technique.

The lipid vesicles prepared may be obtained in 20 a defined size range by various techniques. Methods for reducing size heterogeneity in small unilamellar vesicles by gel filtration and ultra centrifugation have been described. A method of reducing the size and the size heterogeneity of lipid vesicles by extrusion 25 through polycarbonate filters having selected pore sizes is described in reference 2. The latter method is advantageous because of its simplicity and because essentially all of the vesicles are recovered, the larger ones being converted to desired-sized smaller 30 vesicles by passage through the filter.

It can be appreciated from the foregoing that lipid vesicles having a desired size range, morphology, deformability, fluid mobility and surface charge and reactivity characteristics may be prepared by proper 35 selection of lipid components and preparative techniques.



-9-

### Enzyme and Ligand Coupling to Lipid Vesicles

This section is concerned with techniques used to couple ligand and enzyme molecules covalently to surface lipids in lipid vesicles. The ligand molecules in the reagent function to bind the reagent to anti-ligand binding sites on a separable support. As used herein, the term "ligand" refers broadly to either species in a binding pair composed of a target molecule having one or more specific epitopic features, and a target-binding molecule which recognizes such features to bind the target molecule specifically and with a high affinity. "Anti-ligand" refers to the other of the two species in the binding pair. Among the binding pairs which are contemplated by the present invention are antigen-antibody, immunoglobulin-protein A, carbohydrate-lectin, biotin-avidin, hormone-hormone receptor protein and complementary nucleotide strands. More generally, the ligand may include any fragment or portion of a ligand molecule which is capable of participating with the opposite member of the pair in specific, high affinity binding. For example, in an antibody-antigen pair, the binding ligand may include the antigen binding  $F(ab')_2$  or  $Fab'$  fragments. As another example, in the protein A-immunoglobulin pair, the target ligand may include only the  $Fc$ .immunoglobulin fragments. According to an important feature of the present invention, relatively impure ligand mixtures containing as little as 0.5 to 20 mole percent of specific ligand molecules may be employed in the vesicle reagent.

The enzyme in the reagent includes an enzyme which can function to produce a measurable enzyme activity in the presence of suitable substrate(s) and necessary cofactor(s) with the enzyme covalently to the outer surface of a lipid vesicle. Preferably the enzyme



-10-

can be obtained in pure or near-pure form and is relatively stable on storage in solution, or is resistant to freezing or lyophilization. For most applications, enzymes whose activity can be expressed by an easily detectable color change will be preferred. Representative classes of enzymes contemplated herein include oxidoreductases, typified by luciferase, glucose oxidase, galactose oxidase and catalase; hydrolases, typified by various types of phosphatases; glycoside hydrolases, such as beta-galactosidase; peptidases; and lyases.

One enzyme which has been used advantageously in the reagent of the invention is beta-galactosidase derived from a bacterial source. Among the advantages of this enzyme are: (1) the enzyme is available in purified form with high specific activity; (2) the enzyme contains free thiol groups that can be joined to reactive lipids without affecting the enzyme activity; and (3) both fluorogenic and chromogenic substrates are available. The relatively low molecular weight of the enzyme with respect to lipid vesicles allows for the attachment of a relatively large number of enzyme molecules on each vesicle, as will be seen below.

Two or more enzyme species may be attached to the lipid vesicles in accordance with the present invention. The plural enzymes may function independently, or cooperatively, as where the product generated by one enzyme is used as the substrate by another.

Several methods are available for coupling biomolecules covalently to the polar head groups of lipids. As a general consideration, it is important to select a coupling reaction which does not significantly reduce the enzymatic activity or ligand binding activity of the molecules being coupled. At the same time it is important to select a method which produces a relatively



-11-

high coupling efficiency. In this regard, where the enzyme and ligand components are coupled to the vesicles in simultaneous reactions, the relative reactivities of the two species toward the lipid sites must be taken  
5 into account. Finally, care must be exercised to avoid reactions which would produce significant cross linking of the vesicle lipid components to each other, or of the individually - coupled ligand or enzyme molecules to one another, since any cross linking of the reagent  
10 components (except for the individual lipid/surface component conjugations) would reduce the fluid mobility of the surface lipids and attached molecules. Without intending to limit the scope of the invention, two preferred methods of coupling biomolecules, particularly  
15 proteins, to lipid vesicles will be described herein.

The first method involves Schiff-base formation between an aldehyde group on the lipid or molecule to be coupled, and a primary amino group on the other of the two reactants. The aldehyde group is preferably formed  
20 by periodate oxidation. The coupling reaction, after removal of the oxidant, is carried out in the presence of a reducing agent. Although the nonlipid molecule being coupled may be oxidized, more commonly it is the lipid group which is the aldehyde precursor since  
25 periodate treatment inactivates many proteins. Typical aldehyde-lipid precursors include lactosylceramide, trihexosylceramide, galactocerebroside, phosphatidylglycerol, phosphatidylinositol and gangliosides.

30 In practice, the vesicles are oxidized by periodate for a period sufficient to produce oxidation of a majority of the oxidizable lipid groups, and thereafter the vesicles are separated from the periodate by column gel filtration. Aldehyde groups on the  
35 vesicle surfaces are conjugated with a primary amine, such as a lysine group in a protein, to form a Schiff's



-12-

base which is subsequently reduced with sodium boro hydride or sodium cyanoboro hydride to form a more stable bond. Typically, for conjugation reduced with sodium boro hydride, oxidized lipid vesicles at a 5 concentration of between about 5 and 10 micromoles of total lipid are mixed in 1 ml with 10 to 30 milligrams of protein at an alkaline pH. The reaction is carried out for about 2 hours at room temperature. For conjugation reduced with sodium cyanoboro hydride, the 10 reaction typically is carried out over longer reaction times. The reader is referred to reference 3 for additional details.

Using lipid vesicles prepared by reverse phase evaporation and extruded through a 0.2 micron pore-size 15 polycarbonate membrane, up to about 200 micrograms of immunoglobulin G (IgG) per micromole of lipid vesicle lipid can be attached to the vesicle surfaces by the above method. Based on a calculated number of about  $1.2 \times 10^{12}$  vesicles per micromole of vesicle lipid, this 20 conjugation ratio corresponds to about 600 IgG molecules per lipid vesicle. Studies conducted in the support of the present application indicate that correspondingly smaller molecules can be coupled to lipid vesicles in correspondingly larger numbers. Thus, up to about 1800 25 Fab' antibody fragments per lipid vesicle (in the 0.2 micron diameter range) can be attached. The method has wide applicability, due to the general availability of primary amine groups in proteins and other biomolecules which can be reacted with oxidation-produced aldehydes 30 in selected lipids.

A second general coupling technique is applicable to thiol-containing molecules, involving formation of a disulfide or thioether bond between a vesicle lipid and the molecule attached. The technique 35 is particularly useful for coupling  $F(ab')_2$  and Fab' antibody fragments to lipid vesicles.



-13-

In the disulfide interchange reaction, phosphatidylethanolamine is modified to provide a pyridylthio derivative which can react with an exposed thiol group in a protein or other biomolecule. The 5 reader is referred to reference 4 for a detailed discussion of reaction conditions used in the method. As reported there, a coupling ratio of up to 600 micrograms of Fab' antibody fragments per micromole of phospholipid can be achieved. Based on calculations 10 similar to those presented above, this number corresponds to about 6000 Fab' antibody molecules per 0.2 micron diameter vesicle.

The thioether coupling method, which is described in detail in reference 5, is carried out by 15 incorporating in the lipid vesicles a small proportion of a sulhydryl-reactive phospholipid derivative, such as N-(4 (p-maleimidophenyl) butyryl) phosphatidylethanolamine (MPB-PE). The lipid vesicles are reacted with a thiol-containing protein to form an 20 essentially irreversible thioether coupling between the protein thiol group and the MPB-PE maleimide group. It is noted that the requisite protein thiol group may be endogenous to the protein or may be introduced on the protein by amino-reactive thiol groups according to 25 known methods. Coupling ratios of up to about 350 mg of sulfhydryl containing protein per micromole of lipid vesicle phospholipid have been obtained.

It is also contemplated herein that enzyme or ligand molecules can be separately and individually 30 attached to lipid vesicles by first coupling the molecules covalently to free lipids dispersed in a detergent solution. The lipid-enzyme or lipid-ligand couples are then incorporated into lipid vesicles, either during vesicle formation or by diffusion into 35 preformed vesicles according to known techniques.

Alternatively, the ligand itself may contain an



-14-

endogenous hydrophobic region --for example, a hydrophobic stretch of amino acids-- by which the ligand can be incorporated into the surface of a lipid vesicle. As an example, it has been shown that human 5 transplantation antigens can be attached to egg lecithin vesicles by anchoring hydrophobic peptide regions in the antigens to the vesicles (reference 6).

It is further contemplated that immunoglobulin or immunoglobulin fragment ligand molecules can be 10 attached to lipid vesicles through pair-specific binding to anti-immunoglobulin antibodies, or fragments thereof, or to protein A covalently attached to the vesicles.

The enzyme and ligand molecules may be coupled to (or incorporated into) lipid vesicles either 15 sequentially, in separate coupling reactions, or in simultaneous reactions. Sequential coupling is indicated where different reactions are used to couple enzyme and ligand molecules to the lipid vesicles, or where the relative conjugation reactivities of the two 20 species is difficult to control. The latter problem may arise, for example, where the reactivity of either species varies significantly during the reaction period.

In one typical protocol, ligand or a 25 ligand-containing mixture, is first reacted by an above-described coupling reaction, with vesicles to produce a desired vesicle surface concentration of the analyte-specific ligand. After the initial coupling reaction has been completed, the vesicles are separated from unreacted ligand molecules, then reacted with 30 enzyme molecules. One advantage of the present invention is that the vesicles may be easily separated from the unreacted solution components of the coupling reaction(s) by centrifugation, facilitating intermediate purification steps that may be required during reagent 35 preparation.

The ratio of analyte-specific ligand to enzyme



-15-

molecules coupled to the vesicles is generally selected to maximize the signal-to-noise ratio in an enzyme immunoassay employing the reagent. Studies on the kinetics and specificity of vesicle reagent binding to various types of separable supports suggest that two countervailing factors are important in maximizing reagent performance. On one hand, a minimum surface concentration of analyte-specific ligands is required to effect stable reagent binding to a support. Binding affinity generally increases as the surface concentration of ligand molecules increases from an average of about 10-15 molecules per vesicle up to about 50-100 ligand molecules per vesicle (of average diameter of about 0.2 microns). On the other hand, as the number of ligand molecules bound to the lipid vesicles is increased, particularly where the ligand fraction coupled to the vesicle is relatively impure, the number of sites available for enzyme attachment to the vesicles is reduced. Ideally, as in the case where a relatively pure ligand preparation is coupled to the lipid vesicles, the vesicles can easily accomodate 100 or more ligand molecules and several times that number of enzyme molecules.

Binding studies done in support of the present application indicate that at least two, and probably three or more reagent ligand molecules must bind specifically to the separable support binding sites in order to produce stable attachment of the vesicle to the support. The surface concentration of ligand molecules required to promote such stable multi-site vesicle binding to a macromolecular support can be quite low, on the order of about 15 molecules per vesicle. This feature is believed to be due to in part to the highly mobile, or fluid nature of the lipid-bound surface molecules on the reagent vesicle surfaces. Diffusion constants of the order of  $10^{-11}$  to  $10^{-9}$   $\text{cm}^2/\text{sec}$  for



-16-

phospholipid diffusion within lipid bilayers have been measured (reference 7).

Binding efficiency may be further enhanced where the binding sites on the solid support are 5 themselves carried on lipid vesicles. Here the combined mobility of the ligand molecules on the reagent lipid vesicles and the binding site molecules on the separable support lipid vesicles would facilitate multi-site binding at low ligand and anti-ligand (binding site) 10 surface concentrations.

Another important advantage of the reagent invention is the relatively high surface packing of covalently attached molecules which is achievable in the reagent vesicles. As noted above, it is possible to 15 attach up to several thousand protein molecules on a vesicle surface, based on a protein molecular weight of around 50,000 and a vesicle diameter size of about 0.2 microns. Thus a 0.2 micron vesicle having an average of about 50 Fab' fragments carried on its outer surface can 20 carry nearly 100 times that number of an enzyme having about a 50,000 molecular weight.

Alternatively, where the analyte-specific ligand being coupled to the vesicles constitutes as little as one percent of the total non-enzyme molecules 25 attached to the surface, each vesicle can still accomodate up to several hundred or more enzyme molecules, producing a vesicle whose enzyme to ligand molar ratio is still substantially greater than one. Other advantages of the instant reagent in an enzyme 30 immunoassay will be considered below.

#### Assay Methods

The method of the invention comprises reacting a liposome surface reagent with a separable support carrying analyte-related binding-site molecules. The 35 reagent binds to the support in proportion to the amount of analyte present.



-17-

As used herein, separable support refers to any support structure capable of being readily separated -- for example, by differential centrifugation, precipitation or electrophoretic separation -- from analyte and vesicle reagent components not bound to the support. Another feature of the support is that binding-site molecules can be attached to its surface. The separable support may include a water-insoluble solid support, such as one formed of glass, cellulose, agarose, polystyrene and the like. Macromolecular tissue homogenate structures, intact cells, and cell membrane structures are other contemplated supports. Also as detailed above, the support may include surface-bound lipid vesicles to which the support binding site molecules are attached.

The analyte-related binding site molecules on the support are selected to bind specifically to the reagent ligand molecules, or to the analyte molecules, or to both, depending on the type of enzyme immunoassay method, as considered below. The binding site molecules may be adsorbed to the solid support, or may be covalently attached thereto by means of a suitable coupling reaction which may involve the use of conventional linking agents such as glutaraldehyde. Methods of forming solid supports having a wide variety of attached molecules, being well known to those skilled in the art, will not be detailed herein.

Some types of solid supports are known to have surface irregularities, such as cavities or crevices, which may be inaccessible to reagent liposome particles of the type contemplated herein. The fewer binding sites on the support available for liposome binding can result in a proportionate reduction in assay sensitivity. Further, the solid support may have surface properties which tend to promote non-specific attachment of the liposome reagent, leading to a



decreased signal-to-noise ratio in the assay.

The two problems just mentioned may be reduced or eliminated by employing a solid support in which the binding site molecules are carried on lipid vesicles 5 which are themselves attached to the support. Two representative methods by which lipid vesicles can be attached to solid supports will now be described.

In a first method, glass surfaces, for instance glass tubes or controlled-pore glass beads, are 10 derivitized with glycerol, activated with carbonyldiimidazole and converted into amino-glass by reaction with excess diamino alkane. The amino-glass is converted into pyridyl dithio glass by reaction with N-succinimidyl 3-(2-pyridyldithio) propionate. The 15 pyridyl dithio glass is then reduced with dithiothreitol or 2-mercaptoethanol to yield a glass surface with thio functions.

To achieve reversible attachment of liposomes, the lipid vesicles, prepared to include 20 N-(3-(2-pyridyldithio)propionyl)phosphatidylethanolamine (PDP-PE) synthesized according the method described in reference 4, are reacted with the thio glass at a pH between about 7.0 and 8.5. The disulfide bond which forms between the glass and the vesicles can be cleaved 25 by mild reduction, for example with dithiothreitol at low pH.

Irreversible attachment of vesicles to a glass support may be achieved by reacting the thio glass with lipid vesicles prepared to contain MPE-PE, as described 30 in reference 5, to form a thioether linkage.

It is also contemplated that lipid vesicles may be attached to a solid support noncovalently through specific, high affinity ligand/anti-ligand binding. As one illustration, avidin molecules are attached 35 covalently to a solid support using conventional methods. Lipid vesicles prepared to contain



-19-

biotinylated surface lipids then bind with high affinity to the support. Binding site molecules may be attached to, incorporated into, or formed with the lipid vesicles, according to above-described techniques.

5        Attachment of binding site molecules to lipid vesicles carried on a solid support may increase the accessibility of the liposome reagent to the binding sites on the solid support. Another advantage inherent in this approach is that the vesicles to which the  
10      binding site molecules are attached may themselves be prepared to have a selected surface charge character, with respect to the reagent liposome particles, for enhancing specific binding, and reducing non-specific binding, between the liposome reagent and the solid  
15      support. Because the binding site molecules are themselves supported in highly mobile vesicle-surface arrays, reagent binding to the solid support may be facilitated.

20      Three general types of enzyme immunoassay tests employing a solid support in conjunction with the reagent vesicles will now be described. In a first type of test, the reagent vesicles carry analyte or analyte-like ligands which compete with analyte in solution for binding to anti-analyte binding sites on  
25      the solid support. The analyte, and the ligand attached to the liposome reagent, may be a target-type antigen which compete for binding to an anti-analyte attached to the solid support, or the analyte may be a binding protein which competes with binding proteins on the  
30      reagent for binding to target-type binding sites on the solid support. In both instances, the amount of liposome reagent binding to the solid support varies inversely with the amount of analyte present.

35      The assay reaction is carried out in a suitable reaction medium which may include a biological specimen



-20-

fluid, such as serum, containing the analyte. The pH of the reaction medium is one which is compatible with ligand/anti-ligand binding reactions, and preferably between about 5 and 9. More specifically, the pH and/or 5 ionic strength of the reaction medium may be adjusted to achieve a desired charge interaction between the liposome reagent and the solid support. Generally it can be said that the greater the charge repulsion 10 between the reagent and the support, the less the reagent will bind both specifically and nonspecifically, to the support. It is often an advantage to carry out the binding reaction at a pH and ionic strength which minimizes charge repulsion between the support and the reagent, and to remove nonspecifically-bound reagent 15 later by a washing step, usually with a low-ionic strength, high pH washing solution.

The reaction medium may also be adjusted to have a specific gravity which approximates the buoyant density of the reagent particles. Typically, this can 20 be achieved in a medium having a specific gravity between about 1.0 and 1.2. The adjustment in specific gravity, by reducing the tendency of the vesicles to float or sink in the medium, promotes the requisite contact between the vesicles and the solid support. 25 Alternatively, it may be advantageous in some assay methods to employ a liposome surface reagent which is either more or less dense than the reaction medium, to facilitate separation of the liposome reagent from the support, or to achieve some other advantage related to 30 reagent partitioning.

Sensitivity in the assay requires reacting a defined amount of solid support with a known, selected amount of the liposome reagent. With too little 35 liposome reagent added to the reaction mixture, the binding sites on the solid support can accomodate a substantial quantity of bound analyte without any



-21-

observed analyte-dependent displacement of the liposome reagent from the support. With too much liposome reagent added, excess unbound liposome reagent competes with the analyte for binding to any displaced binding sites on the support. The assay background also tends to be high with too much liposome reagent, due to nonspecific binding to the solid support and excess reagent in the liquid phase. In most assays, the optimal amount of liposome reagent is determined by titrating a given amount of solid support with liposome reagent to an end point which just indicates saturation or near saturation of the support binding sites.

The method can be carried out as a single reaction in which the solid support, a defined amount of liposome reagent, and the analyte are coincubated for a period sufficient to produce binding equilibrium among the reaction components. Typical reaction times range from about 5 minutes to several hours, at temperatures ranging preferably from about room temperature up to 20 37°C or somewhat higher.

Alternatively, the assay may be performed as a two-step reaction in which the analyte is reacted first with the solid support alone, after which the separated solid support is reacted with the liposome reagent. The 25 two-step test may be advantageous where the volume of original solution to be assayed is quite large, or where that solution contains substrates or inhibitors of the liposome reagent enzyme. The two-step reaction also has the advantage that the second reaction in which the 30 liposome particles bind to the solid support can be carried out in a selected reaction medium having a desired pH, ionic strength and specific gravity.

Upon completion of the assay reaction, the 35 solid support is separated from the liquid phase of the reaction medium, including the unbound suspended liposomes, and the support or the liquid phase, or both,



-22-

are assayed for enzyme activity. To reduce the level of non-specifically bound liposome reagent, the separated support is preferably washed one or more times with a washing solution whose pH and ionic strength act to increase charge repulsion between the solid support and the liposome reagent.

In a second type of enzyme immunoassay contemplated herein, the solid support carries an analyte or analyte-like binding molecule which competes with the analyte for binding to the ligand on the liposome reagent. The analyte, and the binding-site molecules on the support may be either a target-type antigen which compete for binding to a target-binding ligand on the liposome, or the analyte may be a target-binding molecule which competes with the solid support for binding to an antigen-like ligand on the liposome reagent. Various considerations relating to the pH, ionic strength and specific gravity of the reaction solution which have been discussed above are applicable to the instant method. Likewise the procedure used for optimizing the amount of liposome reagent added to a given amount of solid support is similar to that already discussed.

A third general assay type is a sandwich technique in which the liposome reagent is bound to a support through a multivalent analyte. The assay is preferably performed as a two-step method in which analyte is first reacted with the support, after which the separated support is reacted with the liposome reagent. The analyte may be either an antibody or an antigen, with the solid support and liposome reagent each carrying an opposite binding pair of the analyte. One advantage of this method is that the amount of liposome reagent bound to the solid support is directly proportional to the amount of analyte present. The method can thus be used to detect very small quantities.



-23-

of analyte. Additionally, since the liposome reagent is bound to the solid support through analyte sandwiching, the amount of liposome added to a given quantity of solid support can be less than the saturating or near saturating amounts required in the previously described tests. In turn, the lower concentration of liposome reagent leads to an improved signal-to-noise ratio in the test.

10 The considerations relating to pH, ionic strength and specific gravity in the reaction medium are similar to those discussed above and will not be described further here.

15 One aspect of the invention which can be appreciated from the above is the provision of an immunoassay kit which includes the liposome reagent of the invention. Also included in the kit is a separable support of the type described above, having surface-attached, analyte-related binding site molecules, which may be directly bound to the support or 20 attached to the support surface through lipid vesicles, as described above. The binding site molecules may be analyte or analyte-like molecules which compete with the analyte for binding to anti-analyte ligand molecules in the surface reagent. Alternatively, the binding site 25 molecules may be an anti-analyte species, where the analyte and analyte-like reagent ligand compete for binding to the support, or where the reagent is bound to the support in sandwich fashion through the ligand.

30 The assay methods just described are intended for detecting the presence or concentration of a free analyte in a sample solution. According to another aspect of the invention, the reagent is used in an enzyme immunoassay for determining the presence or concentration of cell-specific surface antigen 35 analytes. Here the separable support includes a biological cell, and more specifically, a cell membrane



whose outer surface carries the antigen. Typical analytes include blood-type specific antigens carried on the surface of blood cells, species and strain specific surface antigens carried on the surface of various 5 animal tissues, and surface antigens characteristic of particular cellular transformation states in various tissues or in tissue culture. Alternatively, the analyte may include anti-cell surface antigen antibodies attached to the cell by incubation with the free 10 antibody.

In the cell-surface antigen assay, the cell sample to be assayed is added to defined amounts of liposome reagent having analyte-recognition molecules. After a suitable reaction time, under reaction 15 conditions which may be selected in accordance with the considerations mentioned above, the cells and bound liposome reagent particles may be separated from the unbound liposomes which remain suspended in solution by differential centrifugation. The separated cells are 20 then washed to remove non-specifically bound liposomes and the enzyme activity associated with the washed cells is determined. Other antigen-bearing supports, such as viral particles, spores, tissue structure, or other suspendible particulate matter which can be separated 25 readily from unbound reagent and soluble components --for example by differential centrifugation or precipitation-- are also contemplated herein.

From the foregoing, it can be appreciated how the liposome reagent of the invention contributes to the 30 improved signal-to-noise ratio achievable in various types of enzyme immunoassay tests. A high signal level is achieved by virtue of the large number of enzyme molecules which "report" each binding event in the assay. As seen, up to several thousand enzyme molecules 35 can be attached to a vesicle which binds to a support through a small number of binding sites on the support.



-25-

The fact that the enzyme whose activity is being measured is bound to a liposome surface may further enhance the signal level. Several enzymes are known to have increased activity in an immobilized state, a 5 phenomenon thought to be related to favorable surface reaction kinetics. Immobilized enzymes are often less susceptible to inactivation as well.

The noise level in the assay methods described is reduced by limiting non-specific binding of the 10 liposome reagent to a separable support. This may be accomplished, according to the invention, by reacting the liposome reagent with a solid support under pH and ionic strength conditions which favor specific binding between the two, followed by exposing the separated 15 support to a washing medium which removes non-specifically bound liposomes through a charge repulsion effect.

The improved signal-to-noise ratio is observed where the ligand molecules in the reagent represent only 20 a small fraction of the total non-enzyme molecules carried on the reagent vesicles, and where the liposome reagent carries two or more distinct types of ligand molecules or more than one type of enzyme.

The following examples describe particular 25 embodiments of making and using the invention.

Example I

Lipid Vesicle Preparation

The following procedure was used to produce a suspension of lipid vesicles containing the 30 sulfhydryl-reactive phospholipid derivative MPB-PE. The synthesis of MPB-PE was performed substantially as described in reference 5. Briefly, transesterified egg PE was reacted with freshly distilled triethylamine and succinimidyl 4-(p-maleimidophenyl) butyrate in anhydrous 35 methanol under an argon atmosphere at room temperature for two hours. The MPB-PE formed was purified by



-26-

silicic acid chromatography.

Large, unilamellar vesicles were prepared by a reverse phase evaporation method described generally in references 1 and 2. Cholesterol (10 micromole), 5 phosphatidylcholine (9.5 micromole), MPB-PE (.5 micromole) and a trace amount of tritiated dipalmitoylphosphatidylcholine were dissolved in 1 ml of diethyl ether. A buffer, at pH 4.5, containing 20 mM citric acid, 35 mM disodium phosphate, 108 mM sodium 10 chloride and 1 mM EDTA was added (300 microliter), and the two phases emulsified by sonication for one minute at 25°C in a bath sonicator. Ether was removed under reduced pressure at room temperature and the resulting dispersion was extruded successively through 0.4 micron 15 and 0.2 micron Unipore polycarbonate membranes (Biorad Laboratories, Richmond, CA).

The lipid vesicle preparation was examined by electron microscopy. Most of the vesicles were in the 0.2 micron diameter size range and had one or a few 20 bilayer lamellae. Based on the microscopic examination of the vesicles, and the known lipid concentration thereof, a vesicle concentration of about  $1.2 \times 10^{12}$  vesicles per micromole of lipid was calculated.

Example II

25 Coupling of Fab' Fragments to Vesicles

This example examines optimal conditions for coupling antibody Fab' fragments to lipid vesicles formed in accordance with Example I.

30 Rabbit anti-human immunoglobulin G (IgG) antibodies were isolated and purified according to conventional methods.  $F(ab')_2$  dimers were prepared by pepsin digestion of the purified antibodies. The dimer fragments were reduced with dithiothreitol at a pH of about 4.8 to produce Fab' monomer fragments. The pH of 35 the reduction reaction is important in that when the reaction is performed significantly above pH 4.8, (i.e.,



-27-

pH 5.0) overreduction may occur which leads to inactivation of the antibody fragments, while underreduction, which may occur which at a lower pH (i.e., pH 4.5), is characterized by relatively poor 5 coupling efficiency to the vesicles.

Freshly prepared vesicles at a concentration of about 1 micromole of phospholipid per ml were reacted with freshly prepared Fab' fragments at a concentration selected between 0.5-4.0 mg per ml. The reaction was 10 carried out in a pH 6.5 buffer under a stream of argon for up to 12 hours at room temperature. The vesicles were separated from unconjugated antibody fragments by differential centrifugation. The amount of protein conjugated to the vesicles varied according to the 15 initial concentration of antibody fragments in the reaction. At an initial protein concentration of 4 mg/ml, approximately 500 micrograms of Fab' per micromole lipid were coupled to the vesicles in eight hours. For vesicles in the 0.2 micron diameter size, 20 this corresponds to about 5,000 molecules of Fab' monomer fragments per vesicle. This number is somewhat higher than that reported in the literature and may be due in part to the reducing conditions used to reduce F(ab')<sub>2</sub> dimers to Fab' monomers.

25 To test the specificity of binding of the Fab'-liposome reagent, Rh+ human red blood cells were sensitized with human anti-D IgG and incubated with the anti-IgG carrying vesicles. Vesicle concentrations between 10 and 125 nanomoles of phospholipid per ml were 30 mixed with an equal volume of a 2 percent suspension of the sensitized erythrocytes. The erythrocytes were incubated with the reagent for two hours at room temperature, after which they were separated and washed by low speed centrifugation in a clinical centrifuge. 35 Radioactivity associated with the cell-bound vesicles increased quantitatively with increasing amounts of the



liposome added, up to a saturation point corresponding to about 5,000 liposome vesicles per red blood cell. Binding of liposomes to human erythrocytes not coated with human IgG was less than about 5 percent of that of antibody-specific liposome binding.

Example III

Coupling Fab' Fragments and Beta-Galactosidase to Lipid Vesicles

This example demonstrates the effect of lipid vesicle surface charge on the relative amounts of coupling of Fab' fragments and beta-galactosidase to lipid vesicles.

Lipid vesicles were prepared according to the procedure described above, except that the vesicles were prepared to contain either 10 percent or 20 percent phosphatidylglycerol and proportionately less phosphatidylcholine.

Purified rabbit anti-human IgG antibodies were pepsin-digested and reduced with dithiothreitol for twenty minutes at pH 4.8, according to the method above. Beta-galactosidase (847 IU/mg), was obtained from Boehringer-Mannheim.

Enzyme and freshly reduced ligand preparations were reacted with one of the two lipid vesicle preparations under reaction conditions similar to those described in Example II. Specifically, 1 mg per ml Fab' and 0.25 mg per ml beta-galactosidase were reacted with 1 micromole vesicle lipid in one ml reaction buffer containing 20 mM citric acid, 35 mM disodium phosphate, 108 mM NaCl, and 1 mM EDTA, adjusted to pH 6.5 with 1 N NaOH. The reaction was stirred under a stream of argon for 14 hours at room temperature. The vesicles were twice pelleted by centrifugation at 20,000 G for 20 minutes to remove unreacted proteins.

The pelleted and resuspended reagent vesicles were assayed for beta-galactosidase activity using the



-29-

chromogenic substrate ortho-nitrophenol-3-D-galactopyranoside (nitrophenyl galactoside). The relative specific activity values shown in Table I represent, respectively, 54 percent and 64 percent of the total beta-galactosidase activity added to the coupling reaction mixture that was found to be associated with the vesicles. The data indicate greater enzyme coupling reactivity toward the vesicle preparation containing the higher concentration of phosphatidylglycerol (PG), apparently resulting from the greater charge attraction between the negatively charged phosphatidylglycerol surface groups and the positively charged enzyme.

TABLE I

PG	B-Gal	Coupling Efficiency	signal-to-noise ratio
10%	393	54	6.8
20%	431	64	4.8

The specific binding activity of the two reagent preparations toward human IgG adsorbed on a polypropylene solid support was measured to determine roughly the relative amounts of Fab' carried on each of the two vesicle reagents. Polypropylene tubes were coated with human IgG according to known procedures. An aliquot containing 1 nanomole of each vesicle preparation was added to a coated tube, and the suspension was incubated for a half hour at room temperature in a phosphate-buffered saline buffer, pH 7.4. As a control, the two vesicle preparations were also incubated with tubes coated only with bovine serum albumin (BSA). After incubation, the tubes were washed one time with a low-salt buffer and the enzyme activity (color development) associated with each of the tubes was determined. The signal-to-noise ratio for each of the two vesicle preparations was calculated by dividing the enzyme activity obtained for the IgG coated tubes by



-30-

that for the tubes coated only with BSA. As seen in Table I, a greater signal-to-noise ratio, presumably attributable to a greater surface concentration of Fab' molecules, was associated with the vesicle preparation 5 containing less phosphatidylglycerol.

The data in Table I suggest that an increased negative charge on the surface of lipid vesicles favors beta-galactosidase coupling, resulting in enzyme coupling efficiencies of up to about 60 percent. The 10 data also indicate that nanomolar amounts of the liposome reagent are sufficient to produce a strong signal-to-noise ratio in a binding assay, and that the ratio of enzyme and ligand molecules in the vesicles can be adjusted to produce an optimal signal-to-noise ratio.

15

#### EXAMPLE IV

##### Preparation of a Reagent

###### Having Different Ligand to Enzyme Ratios

Reagent vesicles having different ratios of surface attached enzyme and ligand molecules were prepared, and their immunospecific binding to 20 erythrocytes examined.

Lipid vesicles containing MPB-PE thiol-reactive surface groups were prepared in accordance with the method described in Example I.

25

Rabbit anti-human IgG antibodies were obtained in a form purified according to standard methods. Reduced Fab' fragments, and beta-galactosidase were provided in accordance with Example III. Vesicle preparations, containing about 1 micromole of vesicle 30 lipid, were reacted with selected amounts of reduced Fab' and beta-galactosidase, as indicated in Table II. The reactant concentrations of Fab' were 0.75, 1.0 or 1.25 mg per ml (column 1), and those of beta-galactosidase, were 0.1, 0.3 or 0.5 mg per ml 35 (column 2) for each Fab' concentration. The coupling reactions were carried out at pH 6.8 under a stream of



-31-

argon gas for 12 hours at room temperature, similar to what has been described above. The vesicles were twice washed to remove unreacted Fab' and beta-galactosidase, and the protein concentration associated with each 5 vesicle preparation was determined according to the method of Lowry (reference 8). Column 3 in Table II shows the measured protein concentrations, expressed in micrograms of protein per micromole vesicle phospholipid. The data show that, for each reactant 10 concentration of Fab', increasing amounts of beta-galactosidase resulted in increasing amounts of protein covalently coupled to the vesicles. That the increased reagent protein concentration is attributable to increased amounts of coupled beta-galactosidase can 15 be seen from the data in column 4 in Table II, showing specific activities of beta-galactosidase (expressed as arbitrary beta-galactosidase activity units per micromole of vesicle lipid). It is interesting to note that the amount of beta-galactosidase coupled to the 20 vesicle lipids was relatively independent of the initial reactant concentration of Fab' in the Fab' concentration range shown (column 4).

The binding of the 9 different reagent preparations to IgG-coated erythrocytes was determined 25 in accordance with the method described in Example III. Sensitized (IgG-coated) red cells, after incubation with a vesicle reagent, were separated by centrifugation at low speed and washed in a low-salt solution, made 30 isotonic with sucrose, to remove nonspecifically bound reagent. Enzyme activity associated with the red blood cells was used to determine the percent of vesicle reagent which bound to the cells. The values, which are shown in column 5 in Table II, confirm that liposomes having greater immunospecific binding capacity can be 35 prepared by coupling greater amounts of ligand to the vesicle surfaces, and that the amount of ligand bound



-32-

depends both on the initial reactant concentration of ligand, and on the relative reactant concentrations of ligand and enzyme.

TABLE II

5	1	2	3	4	5
	Fab'	B-Gal	Reagent Protein	Reagent B-Gal	Reagent Binding to Cells
10	0.75	0.1	330	51	36
	"	0.3	380	104	20
	"	0.5	545	246	25
15	1.0	0.1	355	29	42
	"	0.3	439	120	30
	"	0.5	500	265	31
20	1.25	0.1	370	50	68
	"	0.3	450	140	52
	"	0.5	565	246	27

## Example V

Enzyme immunoassay to detect human subgroup antibodies on sensitized erythrocytes.

This example illustrates use of the liposome reagent of the invention in an enzyme immunoassay to detect the presence of anti-subgroup antibodies on human erythrocytes, and in particular, anti-D, anti-Jka, and anti-Fya IgG antibodies carried on sensitized erythrocytes.

To prepare the liposome reagent, lipid vesicles containing MPB-PE were prepared in accordance with Example I. Immunopurified anti-human IgG antibodies were prepared and treated as described above to produce Fab' fragments, each of which contains at least 1 thiol group. These fragments (0.75 mg per ml), and beta-galactosidase, (0.4 mg per ml) were reacted with the lipid vesicles (1.0 micromole of phospholipid per ml) for 18 hours at room temperature, pH 6.8. The reagent was separated from unreacted protein by



-33-

centrifugation and resuspended in a suitable reaction buffer.

Erythrocyte samples typed according to either D, Fya or Jka subgroup type were used. To sensitize 5 each cell type, freshly washed cells were subdivided and each sample incubated with one of a series of two-fold serial dilutions of the appropriate anti-D, anti-Fya, or anti-Jka typing sera at 37°C for 30 minutes. The cells were washed three times with a saline solution. D, Fya, 10 and Jka positive control cells were washed three times with the above saline solution and resuspended in phosphate buffered saline. The sensitized cells were treated with liposome surface reagent, (5 nanomoles liposomes per  $5 \times 10^7$  cells) for 30 minutes at room 15 temperature, with rocking every few minutes. The cells were subsequently washed 5 times with saline/BSA, resuspended in 10% sucrose containing the enzyme substrate nitrophenyl galactoside, and incubated for 10 minutes at room temperature. Cells were pelleted and 20 the enzyme activity determined by measuring the spectrophotometric absorption of the supernatant at 405 nm.

A linear relationship between the reagent enzyme activity observed (supernatant absorption) and 25 number of surface specific IgG molecules (antisera dilution) was observed over a wide dilution range for all three cell types. The immunoassay was between about 8 and 32 times more sensitive, in terms of the minimum 30 number of erythrocyte-bound antibodies which were detectable, than a standard anti-globin agglutination test which is used commonly for the determination of erythrocyte subgroup antigens.

Example VI

Enzyme immunoassay for determination of Rubella antibodies

35

A lipid vesicle surface reagent was prepared



-34-

essentially according to the method described in Example IV. Specifically, 0.75 mg per ml of immunopurified anti-human IgG Fab' fragments and 0.4 mg per ml of beta-galactosidase were reacted with lipid vesicles containing MPB-PE, under the reaction conditions described in Example IV.

A control sample containing a known amount of Rubella antibody was prepared in 4 different sample concentrations, namely, an undiluted sample and 1:5, 1:25 and 1:125 serial dilutions thereof. The antibody was then reacted with solid support discs coated with Rubella antigens (Cordis Laboratories) for about 45 minutes at room temperature. The support discs were washed with saline/BSA, then placed in 0.5 ml of a high salt solution containing 250 mM NaCl, 100 mM phosphate, and a 25 microliter aliquot of the liposome surface reagent (0.1 micromoles per ml). The liposome reagent was incubated with the support for about 2 hours at room temperature. The support discs were then washed two times with saline/BSA, and the enzyme activity associated with the discs determined according to the method noted above.

Table III shows the relative enzyme activity, expressed in units per ml, associated with each of the different-concentration samples indicated in the table. The data show increasing levels of enzyme activity associated with increasing amounts of Rubella antibody added to a solid support. The negative control serum contained no Rubella antibody.

Table III

<u>Antibody dilution</u>	<u>B-Gal. activity (OD<sub>405</sub>)</u>
1:0	0.49
1:5	0.38
1:25	0.26
35 1:125	0.16
negative control	0.16



-35-

While the invention has been described with particular reference to specific examples, it will be understood that these examples are in no way intended to limit the scope of the invention. Various changes and 5 modifications may be made without departing from the spirit of the invention.



-36-

## WHAT IS CLAIMED IS:

1. A method for assaying an analyte comprising providing a reagent composed of particles, each having a surface array of laterally mobile analyte-related ligand molecules and enzyme molecules, incubating the reagent in an aqueous reaction medium to produce partitioning of the particles between a separable support and solution phase, according to the concentration of analyte present,  
10 separating the support from the reaction medium, and assaying the enzyme activity associated with particles in one of the two separated phases.
2. The method of Claim 1, wherein the particles include lipid bilayer vesicles in the 0.05 to 10.0 micron diameter size range, each vesicle has in its surface array, molecules of a ligand-specific binding protein covalently attached to its surface, and the ligand molecules include an average of at least about 15  
15 immunoglobulin molecules per vesicle bound to the binding molecules.
3. The method of Claim 1, wherein the particles include lipid bilayer vesicles in the 0.05 to 10.0 micron diameter size range, and each vesicle has an average of at least about 15 ligand molecules covalently attached to its surface.  
25
4. The method of Claim 3, wherein the ligand includes protein A.
5. The method of Claim 3, wherein the ligand molecules include covalently attached antibody or antibody fragment molecules constituting between about 0.5 and 20% of the total number of antibody or antibody fragment molecules covalently attached to the vesicle surface.  
30
- 35 6. The method of Claim 3, wherein the ligand molecules include substantially pure antibody or



-37-

antibody fragment molecules, and the ratio of surface concentration of enzyme molecules to ligand molecules is greater than 2.

7. The method of Claim 3, wherein the ligand 5 molecules include Fab' antibody fragments conjugated to the lipid vesicles through thioether or disulfide bonds.

8. The method of Claim 3, wherein said enzyme includes beta-galactosidase.

9. The method of Claim 3, which further 10 includes adjusting the specific gravity of the reaction medium between about 1.0 and 1.2, to approximate the buoyant density of the particles.

10. The method of Claim 3, wherein both the separable support and the reagent particles include 15 acidic surface groups, and said method further includes exposing the support and reagent particles to a solution pH and ionic strength which produce charge repulsion between the support and the reagent particles.

11. The method of Claim 3, wherein the 20 separable support includes a solid support and the reagent particles compete with analyte in the reaction medium for binding to the support.

12. The method of Claim 3, wherein the 25 separable support includes a solid support and the solid support competes with the analyte for binding to the reagent particles.

13. The method of Claim 3, wherein the 30 separable support includes a solid support defining a surface, other lipid vesicles attached to the support surface, and analyte-related binding site molecules carried on the other lipid vesicle surfaces.

14. The method of Claim 3, wherein the 35 separable support includes a biological-material support, having surface-attached analyte molecules, and the reagent ligand molecules include anti-analyte binding proteins.



-38-

15. The method of Claim 14, wherein the support includes a blood cell type which can be identified according to a cell-specific surface antigen analyte.

5 16. An enzyme immunoassay method comprising preparing lipid bilayer vesicles in the 0.05 to 10.0 micron diameter size range,

covalently attaching to one population of lipid components in each vesicle, an average of at least about 10 15 molecules of an analyte-related ligand,

covalently attaching enzyme molecules to another population of lipid components in each vesicle to produce reagent particles having laterally mobile surface arrays of enzyme and ligand molecules,

15 reacting the reagent particles in an aqueous medium with a separable support to produce particle binding thereto in proportion to the concentration of analyte present,

separating the support from the reaction 20 medium, and

assaying the enzyme activity associated with one of the two separated phases.

17. The method of Claim 16, wherein attaching the ligand and enzyme molecules covalently to the 25 vesicles includes simultaneously reacting selected relative molar amounts of the two molecule species with the vesicles under conditions which produce a desired molar ratio of the two species attached to the vesicle surfaces.

30 18. The method of Claim 16, wherein preparing said vesicles includes selecting a lipid composition which produces in the vesicles, a surface charge which enhances the coupling reactivity of one of the ligand and enzyme molecules with respect to the other.

35 19. The method of Claim 18, wherein the ligand and enzyme molecules are attached to the vesicles in



simultaneous reactions.

20. The method of Claim 16, wherein the ligand includes analyte-related antibody or antibody fragments which constitute between about 0.5 and 20% of the total 5 number of antibody or antibody fragment molecules covalently attached to the reagent vesicles.

21. The method of Claim 16, wherein the ligand includes  $F(ab')_2$  or Fab' fragments which are coupled to the lipid vesicles through thioether or disulfide bonds.

10 22. The method of Claim 21 wherein the ligand includes Fab' fragments which are produced by reduction of  $F(ab')_2$  fragments with dithiothreitol or dithioerythritol at a pH of about 4.8.

15 23. The method of Claim 16, wherein the ligand is substantially homogeneous and the ratio of enzyme to ligand molecules is greater than 2.

24. The method of Claim 16, wherein the enzyme includes beta-galactosidase.

20 25. The method of Claim 16, wherein the support includes acidic groups, the particles are prepared to include acidic surface groups, and said method further includes exposing the support and the reagent particles to a solution pH and ionic strength which produces charge repulsion between the support and 25 the particles.

26. The method of Claim 16, which further includes adjusting the specific gravity of the reaction medium between about 1.0 and 1.2 to approximate the buoyant density of the reagent particles.

30 27. The method of Claim 16, wherein the support includes a solid support, and the reagent particles compete with analyte in the reaction medium for binding to the support.

35 28. The method of Claim 16, wherein the support includes a solid support, and the support competes with the analyte for binding to the reagent



-40-

particles.

29. The method of Claim 16, wherein the support includes a biological-material support having surface-attached analyte molecules, and the reagent 5 ligand molecules include anti-analyte binding proteins.

30. The method of Claim 29, wherein the support includes a blood cell type which can be identified according to a cell-specific surface antigen analyte.

10 31. The method of Claim 16, wherein the support includes a solid support defining a surface, other lipid vesicles attached to the support surface, and analyte-related binding site molecules carried on the other lipid vesicle surfaces.

15 32. An assay reagent adapted to bind specifically to an analyte to be assayed, said reagent comprising

lipid bilayer vesicles in the 0.05 to 10.0 micron diameter size range,

20 an average of at least about 15 molecules of an anti-analyte binding protein attached to one population of lipid components in the outer surface of each vesicle, in a mobile surface array thereon, and

25 enzyme molecules covalently attached to another population of lipid components in the outer surface of each vesicle, to form with said binding protein molecules, an array of surface attached molecules in which the total enzyme activity of the reagent is substantially unaffected by binding of analyte to said 30 binding protein.

33. The reagent of Claim 32, wherein the binding protein includes protein A.

34. The reagent of Claim 32, wherein the binding protein includes immunoglobulin or 35 immunoglobulin fragments which are attached immunospecifically to anti-immunoglobulin antibodies or



-41-

fragments thereof, or to protein A covalently coupled to said one lipid population.

35. The reagent of Claim 32, wherein the binding protein includes analyte-specific antibody or 5 antibody fragments comprising between about 0.5 and 20% of the total number of antibody or antibody fragments covalently attached to the vesicles.

36. The reagent of Claim 32, wherein the binding protein includes substantially pure antibody or 10 pure antibody fragments attached covalently to the vesicles.

37. The reagent of Claim 36, wherein the ratio of enzyme to antibody fragments molecules is greater than 2.

15 38. The reagent of Claim 34, wherein the antibody fragments include Fab' fragments which are covalently attached to the vesicle through thioether or disulfide bonds.

20 39. The reagent of Claim 32, wherein the enzyme includes beta-galactosidase.

40. The reagent of Claim 32, for use with a separable support to which the reagent is adapted to bind in proportion to the amount of analyte present, wherein said vesicles are prepared to have a buoyant 25 density, in combination with surface-attached enzyme and binding protein molecules, substantially equal to the specific gravity of the reaction medium in which the binding of the reagent to the solid support is adapted to occur.

30 41. The reagent of Claim 32, for use with a support having a negative surface charge at a selected pH and ionic strength, wherein the vesicles are prepared to have a net negative charge at such pH and ionic strength.

35 42. The reagent of Claim 41, wherein the support includes a biological-material support having



-42-

surface-attached analyte molecules.

43. The reagent of Claim 42, wherein the support is a blood cell type which can be identified according to a cell-specific surface-antigen analyte.

5 44. The reagent of Claim 32, wherein said support includes a solid support defining a surface, other lipid vesicles attached to the support surface, and analyte-related binding site molecules carried on the other lipid vesicle surfaces.

10 45. An enzyme immunoassay kit for determination of an analyte comprising

a support reagent composed of a separable support having surface attached, analyte-related binding site molecules, and

15 a lipid vesicle reagent composed of lipid vesicles in the 0.05 to 10.0 micron diameter size range, each vesicle having a laterally mobile surface array of enzyme molecules and an average of at least about 15 ligand molecules capable of binding to at least one of 20 said analyte and said binding sites.

46. The kit of Claim 45 in which the ligand molecules includes antibody or antibody fragment molecules.

25 47. The kit of Claim 46, wherein the antibody or antibody fragment molecules comprise between about 0.5 and 20% of the total number of antibody or antibody fragments covalently attached to the reagent.

30 48. The kit of Claim 46, wherein the antibody or antibody fragment molecules attached to the vesicles are substantially homogeneous and the ratio of enzyme to ligand molecules is greater than 2.

49. The kit of Claim 45, wherein the enzyme includes beta-galactosidase.

35 50. The kit of Claim 45 in which the ligand molecules include Fab' fragments covalently attached to the vesicles through thioether or disulfide bonds.



-43-

51. The kit of Claim 45, wherein the support and the vesicle reagent both include acidic groups which are adapted to produce charge repulsion between the support and the reagent at a selected pH and ionic strength.

5 52. The kit of Claim 45, wherein the analyte includes an immunoglobulin which is capable of binding immunospecifically to antigenic binding sites on the solid support, and the reagent ligand includes an 10 immunoglobulin-binding protein.

15 53. The kit of Claim 45, wherein said support defines a surface, and said binding site molecules are carried on the surfaces of second lipid vesicles which themselves are attached to said surface.

54. A method of producing a lipid-vesicle-surface assay reagent for determination of an analyte comprising

preparing lipid bilayer vesicles in the 0.05 to 10.0 micron diameter size range,

20 covalently attaching to one population of lipid components in each vesicle, an average of at least about 15 molecules of an analyte-related ligand, and

25 covalently attaching enzyme molecules to another population of lipid components in each vesicle to produce reagent particles having laterally mobile surface arrays of enzyme and ligand molecules.

55. The method of Claim 54, wherein attaching the ligand and enzyme molecules covalently to the vesicles includes simultaneously reacting selected 30 relative molar amounts of the two molecule species with the vesicles under conditions which produce a desired molar ratio of the two species attached to the vesicle surfaces.

35 56. The method of Claim 54, wherein preparing said vesicles includes selecting a lipid composition which produces in the vesicles, a surface charge which



-44-

enhances the coupling reactivity of one of the ligand and enzyme molecules with respect to the other.

57. The method of Claim 54, wherein the ligand includes  $F(ab')_2$  or Fab' fragments which are coupled to the lipid vesicles through thioether or disulfide bonds.

58. The method of Claim 57, wherein the ligand includes Fab' fragments which are produced by reduction of  $F(ab')_2$  fragments with dithiothreitol or dithioerythritol at a pH of about 4.8.



# INTERNATIONAL SEARCH REPORT

International Application No PCT/US83/02047

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) <sup>3</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC-GOIN 33/54

US- 435/7

## II. FIELDS SEARCHED

### Minimum Documentation Searched <sup>4</sup>

Classification System	Classification Symbols
U.S.	435/4,7,18,174,175,177,188,810 436/503,512,518,519,527,529,530,543,828,829

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched <sup>5</sup>

## III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>14</sup>

Category <sup>6</sup>	Citation of Document, <sup>15</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
Y	US, A, 4,193,983, (ULLMAN), 18 MARCH 1980.	1-58
Y	GB, A, 2,051,360, (DAVIS), 14 JANUARY 1981.	1-58
Y	N, BIOCHEMISTRY, 20, 4229-4238, (1981), FRANCIS MARTIN ET AL, "IMMUNOSPECIFIC TARGETING OF LIPOSOMES TO CELLS: A NOVEL AND EFFICIENT METHOD FOR COVALENT ATTACHMENT OF FAB' FRAGMENTS VIA DISULFIDE BONDS".	7,21,22,38, 50,57,58
Y	US, A, 4,287,300, (GIBBONS), 01 SEPTEMBER 1981.	10,18,25,41, 51,56
A	US, A, 4,130,634, (MOLINARO), 19 DECEMBER 1978.	1-58
A	N, THE JOURNAL OF BIOLOGICAL CHEMISTRY, 257,(1), 286-288, (1982), FRANCIS MARTIN ET AL, "IRREVERSIBLE COUPLING OF IMMUNOGLOBULIN FRAGMENTS TO PREFORMED VESICLES".	1-58
A	N, NATURE, 288, 602-604 (1980), LEE LESERMAN ET AL, "TARGETING TO CELLS OF FLUORESCENT LIPOSOMES COVALENTLY COUPLED WITH MONOCLONAL ANTIBODY OR PROTEIN A".	1-58

- Special categories of cited documents: <sup>15</sup>
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search <sup>9</sup>	Date of Mailing of this International Search Report <sup>9</sup>
20 MARCH 1984	MAR 27 1984
International Searching Authority <sup>1</sup> ISA/US	Signature of Authorized Officer <sup>10</sup> Esther M. Kepplinger ESTHER M. KEPPLINGER

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No <sup>18</sup>
Y	N, CHEMICAL ABSTRACTS, 93: 109734p, 327, (1980), TIMOTHY D. HEATH ET AL, "COVALENT ATTACHMENT OF HORSERADISH PEROXIDASE TO OUTER SURFACE OF LIPOSOMES".	1-58